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# MOJAVE TOXIN:
# A SELECTIVE $Ca^{++}$ CHANNEL ANTAGONIST



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Chloride (-) Sodium (+) Calcium (++)

Mojave toxin, the principal toxic component of the venom of the Mojave rattlesnake, is a protein complex of 22,000 daltons. The mechanism of this potent (LD50 = 0.18 µg/g) neurotoxin is unclear, but physiologic data suggest an action on ion channels. The specificity of Mojave toxin for receptors associated with $Ca^{++}$, $Cl^-$, and $Na^+$ channels was assessed using a competitive binding paradigm. Mojave toxin competitively and irreversibly inhibits binding to dihydropyridine receptors associated with neuronal $Ca^{++}$ channels but had no effect on $Cl^-$ and $Na^+$ channels. The pharmacologic specificity of Mojave toxin makes it a useful probe of $Ca^{++}$ channel function. Neuromuscular Brain Ratty Shaker Electrical Nerve, Nerve transmission, Ligand


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## PREFACE

The work described in this report was authorized under Project No. 21083000B134. This work was started in July 1986 and completed in September 1986. The experimental data are contained in laboratory notebook nos. 86-0070 and 86-0071.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.



The report has been approved for release to the public.

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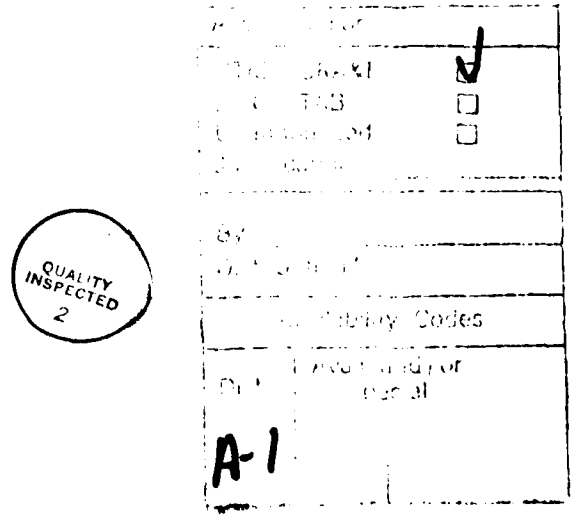

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# MOJAVE TOXIN: A SELECTIVE $Ca^{++}$ CHANNEL ANTAGONIST

## 1. INTRODUCTION

The venom of the Mojave rattlesnake (Crotalus scutulatus scutulatus) has an LD50 in mice of 0.18 µg/g body weight,[1] making it the most potent venom yet extracted from North American rattlesnakes. The principal toxic fraction is known as Mojave toxin (MoTX) and has been determined by gel filtration to be a protein complex of 22,000 daltons consisting of basic phospholipase A2 (PLA2) and an acidic peptide subunit.[2] Toxicologic data are scarce; available studies indicate that mice injected with MoTX display an "inpouching" in the inguinal area with respiratory distress, audible gasping, and prostration within 15 min of injection.[3] Other symptoms include ataxia followed by periods of hyperexcitability, prostration, and tachypnea.

The neural mechanism of MoTX is a matter of conjecture although electrophysiological studies suggest that it acts presynaptically by interfering with stimulus-secretion coupling at the motor endplate.[4] This action suggests a mechanism involving either $Ca^{++}$ channels or the associated $CA^{++}Mg^{++}ATPase$. The recent isolation of a $Ca^{++}$ channel/$Ca^{++}Mg^{++}ATPase$ complex[5] from bovine brain has enabled a direct biochemical analysis of the effects of MoTX on this receptor complex. These studies indicate that MoTX is the only known, naturally occurring $Ca^{++}$ channel toxin other than maitotoxin, blocking 3H-nitrendipine binding to the high affinity dihydropyridine receptor associated with the $Ca^{++}$ channel, as well as stimulating $Ca^{++}Mg^{++}ATPase$ activity.[6] These studies were not able to rule out alterations of nonspecific phospholipase activity as a possible mechanism, nor could they determine the specificity of MoTX for this system.

The present studies were performed to assess the specificity of MoTX for receptors associated with a number of ion channels critical for neurotransmission. These studies were also conducted to determine the reversibility of the effects in those systems where specific interactions were observed. Therefore, competitive binding assays were performed using radiolabelled ion channel ligands for $Ca^{++}$ and $Cl^-$ channels in rat brain and $Na^+$ channels in Torpedo electric organ.

## 2. METHODS AND PROCEDURES

### 2.1 Subjects.

Three hundred and forty-four male, Fischer rats (U.S. Army Medical Research Institute of Chemical Defense Aberdeen Proving Ground, MD) weighing 200-250 g were individually housed in hanging wire cages in an AAALAC accredited facility and fed

laboratory chow (Ziegler Brothers) and tap water ad libitum. The rats were kept under conditions of controlled temperature (24 ± 2 °C) and humidity (30-70%) on a 12-hr light/dark schedule. The animals were allowed to acclimate to their surroundings for at least 1 week prior to the experiments.

2.2 Toxin.

MoTX was provided by Dr. Eppie D. Rael (University of Texas at El Paso, El Paso, TX). The toxin was isolated from the venom of Crotalus scutulatus scutulatus and purified by immunoaffinity chromatography.*

2.3 $Na^+$ Channel Assay.

Frozen electric organ tissue (Torpedo nobiliana, BioFish, Incorporated, Georgetown, MA) was chopped in two volumes of Torpedo buffer (0.154M NaCl, 0.05M Trizma base, 5mM Na2HP04, and 1mM EDTA, ph 7.4). This mixture was homogenized with a Brinkman polytron (setting 5 for 1 min), allowed to rest for 2 min, and homogenized again. The mixture was centrifuged (1000 x g, 10 min, 4 °C, and the supernatants were collected and stored on ice. Pellets were rehomogenized and recentrifuged as before, and the supernatants were combined and centrifuged. Resulting pellets were suspended in one volume of Torpedo buffer. This suspension was homogenized (Wheaton homogenizer, setting 3) with five strokes and kept on ice.

Tissue suspension was incubated for 30 min at 23 °C. Fifty microliters of 3H-[piperidyl-3,4-3H(N)]-phencyclidine (3H-PCP, 2-nM final concentration, 50 Ci/mMol), 50 µL of buffer or a MoTX dilution (1.12-, 2.25-, 11.2- or 22.5-nM final concentration), and 850 µL of Tris buffer (0.05M) were combined in duplicate test tubes. GF/B filters were soaked in poly-l-lysine (50 mg/500 mL) for 30 min at 23 °C. Fifty microliters of tissue was individually added to a tube, which was vortexed, incubated for 30 sec, and aspirated onto a filter disk. Filters were placed in Hang-in scintillation vials; 5 mL of Formula 947 (NEN) was added, and samples were dark and cold adapted and counted in a Packard 300-C liquid scintillation spectrometer.

2.4 $Cl^-$ Channel Assay.

Synaptic membranes were prepared from rat cortical tissue using the Hajos method,[7] and the final pellet was suspended in 5 volumes of Tris/glycerol buffer (10 mM Tris, pH 8.2, containing 16% glycerol).

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*Rael, E.D., Salo, R.J., and Zepeda, H., "Monoclonal Antibodies to Mojave Toxin and Use for Isolation of Crossreacting Proteins in Crotalus Venoms," Unpublished data.

Ten microliters of 35S-tertiarybutylbicyclophosphorothionate (35S-TBPS, 2-nM final concentration, 61.2 Ci/mMol), 100 µL of tissue, 880 µL of $Na^+K^+$ phosphate buffer (10 mM of KH2P04, 10 mM of Na2HP04, and 2 mM of EDTA, added to 150 mM of KCl, pH 6.8), and 10 µL of buffer or MoTX ($9^-$, $90^-$, $180^-$, $540^-$, $900^-$, or 1800-mM final concentration) were combined in duplicate tubes. Contents of the tubes were incubated for 60 min at 23 °C. Thirty minutes before aspirating, GF/B filter strips were soaked in 0.1% polyethylinimine. Contents of the tubes were aspirated onto filter strips using a Brandel tissue harvester and washed twice with 5 mL of $Na^+K^+$ phosphate buffer. Filters were placed in Hang-in scintillation vials to which 5 mL of Formula 963 (NEN) was added, shaken, dark and cold adapted, and counted as before.

2.5 $Ca^{++}$ Channel Assay.

Synaptic membranes were prepared as before, 3H-nitrendipine assays were performed as previously described,[6] and protein concentrations were determined by the Bradford method[8] using bovine serum albumin as standard.

2.6 Reversibility Assay.

Eight hundred microliters of buffer and 100 µL of tissue, prepared as before, were combined in two sets of duplicate tubes. MoTX concentrations (0, 90, 540, and 900 nM) were added to one set of tubes. Contents of tubes were incubated at 23 °C for 60 min. All tubes were centrifuged (20,400 x g, 20 min, 6 °C), resuspended, recentrifuged, and resuspended in buffer to 800 µL for tubes containing no MoTX and to 900 µL for tubes containing MoTX. One hundred milliliters of 3H-nitrendipine (1 nM) was added to all tubes. MoTX concentrations were added to tubes containing only tissue and buffer. All tubes were incubated at 23 °C for 60 min and placed in an ice water slurry for 5-10 min. Two milliliters of Tris/HEPES/20% PEG was added to each tube to terminate the reaction. Contents were aspirated on GF/B filter strips and washed twice with 3 mL of Tris/HEPES/20% PEG. Disks were placed in Hang-in vials with 5 mL of Formula 947, shaken, dark and cold adapted, and counted.

2.7 Data Analysis.

Values for the concentration of MoTX, which produced 50% inhibition (IC50) of specifically bound radioligands, were calculated with the EBDA computer program (EMF Software). Saturation experiments were graphically illustrated by Scatchard plot[9] with Kd and Bmax estimates derived by linear regression.

## 3. RESULTS

### 3.1 $Na^+$ Channel Assay.

MoTX had negligible effects on 3H-PCP binding to Na+ channels in Torpedo tissue over the range of MoTX concentrations tested. In ascending order of MoTX concentration, from zero (control) to high, 3H-PCP binding values were 0.421, 0.451, 0.451, 0.410, and 0.400 fM/µg protein.

### 3.2 $Cl^-$ Channel Assay.

MoTX had negligible effects on 35S-TBPS binding to Cl- channels in rat brain synaptic membranes over the range of MoTX concentrations tested. In ascending order of MoTX concentration, from zero (control) to high, 35S-TBPS binding values were 4.44, 4.12, 3.99, 4.10, 4.14, 4.10, and 4.04 fM/µg protein.

### 3.3 $Ca^{++}$ Channel Assay.

MoTX inhibited 3H-nitrendipine binding to dihydropyridine receptors associated with Ca++ channels in rat brain synaptic membranes with IC50 = 38 nM (Figure). Kinetic analyses of binding experiments performed in quadruplicate indicate a decreased binding affinity in MoTX treated tissue (Kd = 0.95 ± 0.3 nM) compared to controls (Kd = 0.33 ± 0.08 nM) and a decrease in the number of binding sites (Bmax = 61.0 ± 12 pMol/mg protein) compared to controls (Bmax = 84.5 ± 4 pMol/mg protein).

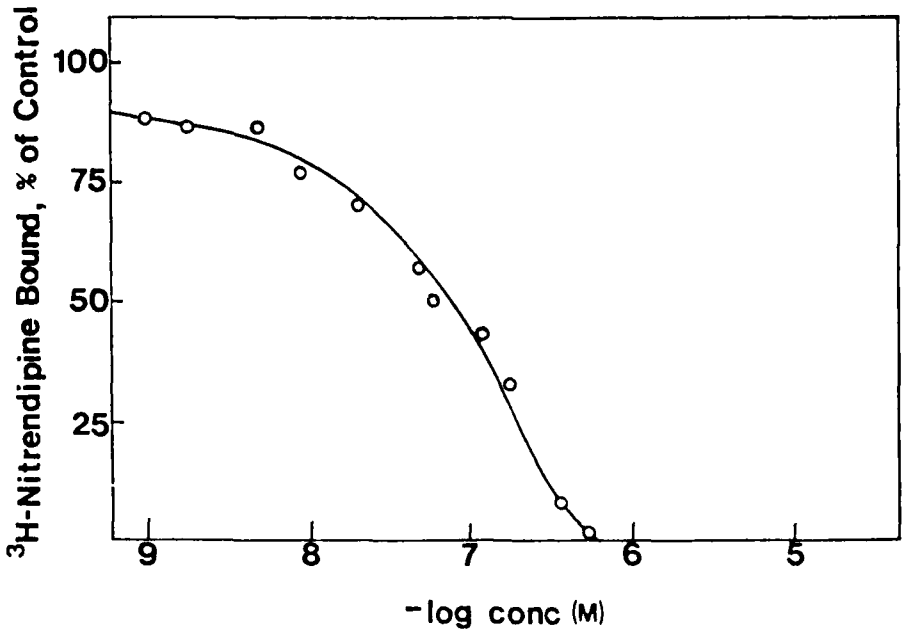


Figure. Competitive Displacement of 3H-Nitrendipine Binding to Rat Synaptic Membranes by Mojave Toxin.

3.4 Reversibility Assay.

The inhibition of high affinity 3H-nitrendipine binding by MoTX was irreversible and, in fact, washing the tissue resulted in a greater degree of inhibition than did MoTX alone (Table).

Table. Reversibility of the Inhibition of 3H-Nitrendipine Binding by Mojave Toxin.

| Toxin Concentration (nM) | 3H-Nitrendipine Bound (pMol/mg protein) | |
|---|---|---|
| | Control | Washed |
| 0 | 95 | 95 |
| 90 | 83 | 84 |
| 540 | 55 | 16 |
| 900 | 50 | 12 |

4. DISCUSSION

MoTX was previously found to inhibit 3H-nitrendipine binding and stimulate $Ca^{++}Mg^{++}$ATPase activity in rat synaptic membranes.[6] These data could not address the issue of specificity of MoTX for this complex, thus limiting the use of this toxin as a $Ca^{++}$ channel probe.

The present study confirms the inhibitory effects of MoTX on 3H-nitrendipine binding, visual inspection of the Scatchard plot indicating that this inhibition is essentially complete over the range of ligand concentrations used. In addition, MoTX had no effect on ligand binding to Cl- channels in brain and Na+ channels in Torpedo, suggesting a selective action on the $Ca^{++}$ channel complex. It follows that because the Cl- and Na+ channels also have lipid requirements, nonspecific phospholipase activity can probably be ruled out as an explanation for the decreased 3H-nitrendipine binding. Similarly, because the Cl- and Na+ channels remain viable, nonspecific proteolytic activity could also be dismissed. The wash data indicate that the inhibition of 3H-nitrendipine binding is irreversible because washing the MoTX treated membranes twice with buffer before performing the 3H-nitrendipine binding assays did not negate the toxin effect. In fact, the wash procedure appeared to potentiate the MoTX effect although no logical explanation for this was possible.

## 5. CONCLUSIONS

The apparent specificity of MoTX for dihydropyridine receptors in rat synaptic membranes suggests that this toxin may be a useful proble of the $Ca^{++}$ channel complex. It is not certain whether MoTX has agonist or antagonist activity; if the former, one hypothesis to explain the stimulation of $Ca^{++}Mg^{++}$ATPase activity by MoTX would be based on the increase in intracellular $Ca^{++}$ resulting from the binding of the toxin to dihydropyridine receptors coupled to $Ca^{++}$ channels. The resolution of this hypothesis will require ion flux studies.

## LITERATURE CITED


1. Tu, A.T., Prescott, B., Chou, C.H., and Thomas, G.J., "Structural Properties of Mojave Toxin of Crotalus scutulatus (Mojave rattlesnake) Determined by Laser Raman Spectroscopy," Biochem. Biophys. Res. Commun. Vol. 68, pp 1139-1145 (1976).

2. Ho, C.L., and Lee, C.Y., "Presynaptic Actions of Mojave Toxin Isolated From Mojave Rattlesnake (Crotalus scutulatus) Venom," Toxicon Vol. 19(6), pp 889-892 (1981).

3. Weinstein, S.A., Minton, S.A., and Wilde, C.E., "The Distribution Among Ophidian Venoms of a Toxin Isolated From the Venom of the Mojave Rattlesnake (Crotalus scutulatus scutulatus)," Toxicon Vol. 23(5), pp 825-844 (1985).

4. Gopalakrishnakone, P., Hawgood, B.J., Holbrooke, S.E., Marsh, N.A., Santanna de Sa, S., and Tu, A.T., "Sites of Action of Mojave Toxin Isolated From the Venom of the Mojave Rattlesnake," Br. J. Pharmac. Vol. 69, pp 421-431 (1980).

5. Chambers, J.P., Wayner, M.J., Rizopoulos, E., Gonzales, M.L., Taylor, R.B., and Valdes, J.J., "Partial Characterization of Two ($Ca^{++}Mg^{++}$)-Dependent ATPase Activities *From Bovine Brain Synaptic Membrane Homogenates*," Brain Res. Bull. Vol. 18, pp 99-107 (1987).

6. Chambers, J.P., Wayner, M.J., Dungan, J., Rael, E.D., and Valdes, J.J., "The Effects of Purified Mojave Toxin On Rat Synaptic Membrane ($Ca^{++}Mg^{++}$)-ATPase and the Dihydropyridine receptor," Brain Res. Bull. Vol. 16, pp 639-643 (1986).

7. Hajos, I., "An Improved Method for the Preparation of Synaptosonal Fractions in High Purity," Brain Research Vol. 93, pp 485-489 (1975).

8. Bradford, M.A., "Rapid and Sensitive Method for the Quantitation of Protein Utilizing the Principle of Protein Dye Binding," Anal. Biochem. Vol. 72, pp 248-254 (1976).

9. Scatchard, G., "The Attraction of Proteins for Small Molecules and Ions," Ann. NY Acad. Sci. Vol. 51, pp 660-672 (1949).